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The objective of the studies described in this report has been to develop a rapid, reliable and simple screening assay of broad scope and sensitivity for applications in the identification of compounds with potential therapeutic value in the treatment of intoxication induced by a variety of sodium channel neurotoxins. The assay should also be able to accomodate the screening of large numbers of samples. These requirements have been met in the form of an in vitro radioligand binding assay in which the binding of a tritiated analog of batrochotoxin serves as a sensitive indicator of test compound interactions at any of at least five different binding domains on the sodium channel. A vesicular preparation from rat cerebral cortex, termed synaptoneurosomes, was selected as a source of mammalian voltage-sensitive sodium channels because of the ease of preparation and the suitability of its biophysical characteristics for the binding assay. Experiments are

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described which delineate conditions and demonstrate the stability of synaptoneurosomes to extended storage periods at -70 C. Set-up of the screening assay may therefore be simplified by bulk preparation of synaptoneurosomes in advance and storage of aliquots for use as required. The assay consists of equilibration of the radioligand and other unlabeled sodium channels neurotoxins with synaptoneurosomes in the absence and presence of test substances at room temperature for 1 hr. Samples are collected by automatic filtration on a 30-place manifold and counted by liquid scintillation spectroscopy. The time required from assay set-up to counting is approximately 2 hr, thus large numbers of samples may be processed easily. Initial testing of the validity of the assay was addressed by evaluating the response of the assay to inclusion of five compounds known to bind to the sodium channel. As anticipated, each of these compounds produced a dose-dependent effect on radioligand binding and in this way were success-fully flagged by the screening assay. One of these compounds, HM-197, a hexahydropy-rimidine derivative, was further investigated by electrophysiological techniques and was found to antagonize the veratridine-induced depolarization of rat phrenic nerve at concentrations that had no effect on sodium channel function when administered alone. These results support the applicability of the screening assay as described and suggest that HM-197 and similar derivatives should be further investigated for their potential therapeutic value. Keywords:

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Development and Testing of an <u>In Vitro</u> Assay for Screening of Potential Therapeutic
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SUMMARY

The objective of the studies described in this report has been to develop a rapid, reliable and simple screening assay of broad scope and sensitivity for applications in the identification of compounds with potential therapeutic value in the treatment of intoxication induced by a variety of sodium channel neurotoxins. The assay should also be able to accomodate the screening of large numbers of samples. These requirements have been met in the form of an *in vitro* radioligand binding assay in which the binding of a tritiated analog of batrachotoxin serves as a sensitive indicator of test compound interactions at any of at least five different binding domains on the sodium channel. A vesicular preparation from rat cerebral cortex, termed synaptoneurosomes, was selected as a source of mammalian voltage-sensitive sodium channels because of the ease of preparation and the suitability of its biophysical characteristics for the binding assay. Experiments are described which delineate conditions and demon trate the stability of synaptoneurosomes to extended storage periods at -70°C. Set-up of the screening assay may therefore be simplified by bulk preparation of synaptoneurosomes in advance and storage of aliquots for use as required. The assay consists of equilibration of the radioligand and other unlabeled sodium channels neurotoxins with synaptoneurosomes in the absence and presence of test substances at room temperature for 1 hr. Samples are collected by automatic filtration on a 30-place manifold and counted by liquid scintillation spectroscopy. The time required from assay set-up to counting is approximately 2 hr, thus large numbers of samples may be processed easily. Initial testing of the validity of the assay was addressed by evaluating the response of the assay to inclusion of five compounds known to bind to the sodium channel. As anticipated, each of these compounds produced a dose-dependent effect on radioligand binding and in this way were successfully "flagged" by the screening assay. One of these compounds, HM-197, a hexahydropyrimidine derivative, was further investigated by elctrophysiological techniques and was found to antagonize the veratridine-induced depolarization of rat phrenic nerve at concentrations that had no effect on sodium channel function when administered alone. These results support the applicability of the screening assay as described and suggest that HM-197 and similar derivatives should be further investigated for their potential therapeutic value.

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FOREWARD

Citations of commerial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978)).

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STATEMENT OF THE PROBLEM UNDER STUDY

The voltage-sensitive sodium channel of excitable membranes is responsible for the fast rising phase of the action potential. The opening of this channel in response to local alterations in the membrane potential is a transient event allowing a momentary influx of sodium ions down an electrochemical gradient, and the channel rapidly inactivates to a distinct closed conformation(s) before returning to the resting state, also under control of the local membrane potential. The sodium channel can therefore be considered to be a conformationally flexible trans-membrane protein with several properties and characteristics that are functionally descriptive and vital, including voltage-dependent activation and inactivation, relative selectivity for sodium ions, and a single channel conductance describing the flux of sodium ions through an open channel. Alteration of any one of these properties can and often does lead to untoward physiological consequences, including death (see e.g.1,2).

Given the central role of the voltage-sensitive sodium channel in impulse propagation of nerve and muscle, it is perhaps teleologically not surprising that Nature has provided an impressive number of naturally-occurring neurotoxins that act specifically and with high affinity at this particular membrane protein. In some instances, such toxins may subserve a predatory function for the bearer (scorpion toxins), while in others their role appears to be strictly defensive (batrachotoxin from the arrow poison frog, *Phyllobates aurotaenia*). In still other cases, it is more difficult to discern a plausible functional relationship between the source of the toxin and effects at voltage-sensitive sodium channels (e.g. the plant alkaloid veratridine). Each of these toxins has attracted the interest of numerous researchers in many laboratories based upon their common property of acting at the sodium channel. The driving force for this research has been primarily the desire to gain information about the structure and function of this vitally important membrane protein. Because of their specificity and high affinity, these neurotoxins have provided the molecular probes necessary for a wide variety of electrophysiological and biochemical studies, culminating in our current understanding of the voltage-sensitive sodium channel.

Perhaps because of the strong focus on their value as biochemical tools, almost no research has been directed towards the development of therapeutic strategies against these compounds as potent sodium channel poisons. It is the objective of the studies to be described here to address the problem of the development of such therapeutic strategies. These compounds represent some of the most potent toxins known, and the ability to intervene effectively in cases of poisoning would of course hold substantial benefit for human health. In addition, it seems clear that this research direction also encompasses the potential to develop new knowledge concerning the sodium channel as well, since agents or conditions which antagonize effects of the neurotoxins may provide further insight into their mechanism of action.

One of the most significant problems to be met in this research concerns the methods which can be applied to disclose potentially therapeutic strategies. It has been stated in reference to the "discovery" of tetrodotoxin that had one intentionally sought to

develop such a unique pharmacological agent through isolation from natural sources or by synthesis, the search almost certainly would have failed (3). Fortunately, the accumulated knowledge of sodium channel pharmacology allows the search for potentially therapeutic compounds to proceed from a stronger footing. This report describes our progress towards the development of a rapid, sensitive screening procedure of broad scope, and its application to the identification of compounds potentially therapeutic against sodium channel neurotoxins.

BACKGROUND AND REVIEW OF THE LITERATURE

The voltage-sensitive sodium channel has a particularly rich pharmacology. Although the individual compounds known to exert significant effects on the channel number over 100 (including synthetic local anesthetics, insecticides, etc.), most of these agents may be grouped into one of five or six classes based on their pharmacological activity and mechanism of action. Neurotoxins in at least three of these classes have been used extensively in the study of the sodium channel. Prominent members of these classes include the heterocyclic guanidines tetrodotoxin (TTX) and saxitoxin (STX), the "lipid-soluble" toxins batrachotoxin (BTX), veratridine, aconitine and grayanotoxin, and the α-polypeptide neurotoxins from scorpion and sea anemone. In the formalism of Catterall (4), TTX and STX bind at site 1 at or near the mouth of the voltage-sensitive sodium channel producing a block of ion flux. The lipid-soluble toxins, of which BTX is the most potent, bind at site 2 causing a shift of the voltage dependence of activation in the hyperpolarizing direction and a concomitant block of the inactivation process, resulting in the stabilization of an open conformation of the channel. These toxins are therefore depolarizing agents. BTX is also known to alter the ionic selectivity and single channel conductance of the sodium channel (5). The α-polypeptide neurotoxins bind at site 3 and act by slowing the process of inactivation. Although these three sites have been the most extensively studied, additional classes of sodium channel agents with distinct binding domains have emerged in recent years. For purposes of this discussion, three additional sites are most relevant. Site 4 binds pyrethroid insecticides which delay channel inactivation and act in synergy with both site 2 and 3 neurotoxins. Site 5 represents the binding domain for local anesthetics which block sodium conductance and allosterically inhibit binding of site 2 toxins. Finally, site 6 binds another class of scorpion polypeptides, the ß-neurotoxins. These compounds shift the voltage dependence of activation, but in a manner distinct from that of the site 2 neurotoxins.

Each of these classes of sodium channel neurotoxins can be associated with a distinct binding domain. As indicated above, however, the binding of toxins from different classes may in fact interact through allosteric mechanisms. In this laboratory we have been most interested in the allosteric relationships affecting the binding of batrachotoxin. Since BTX is known to affect all parameters mediating sodium channel function - activation, inactivation, channel selectivity and conductance (6)- the suggestion is that binding of this class of ligands induces significant conformational perturbations of the channel protein. Conversely, work from this laboratory has demonstrated a remarkable sensitivity of BTX

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binding to conformational changes induced by binding of other ligands at distinct sodium channel domains. These studies were made possible by the synthesis of a tritiated analog of BTX, batrachotoxinin-A benzoate (BTX-B), of high specific activity that retains full biological activity (7).

Using ^{22}Na flux measurements, Ray et al. (8) first demonstrated a positively cooperative heterotropic interaction between α-scorpion toxin from *Leiurus quinquestriatus* (toxin V) and batrachotoxin or veratridine which was adequately accounted for by a two-state allosteric model. This interaction was subsequently confirmed by direct binding measurements in synaptosomes using [^{3}H]-BTX-B (9) and extended to include toxin II from *Anemone sulcata* which shares a common binding domain with α-scorpion toxins (10). Saturating concentrations of scorpion toxin increase the binding affinity of [^{3}H]-BTX-B approximately 15-fold in rat brain synaptosomes (9) and in synaptoneurosomes prepared from either guinea pig or rat cerebral cortex (11). Thus, a prominent interaction between sites 2 and 3 has been firmly established.

Using eletrophysiological techniques, several local anesthetics have been shown to prevent the depolarizing effects of batrachotoxin (12-14). We have confirmed this interaction between sites 2 and 5 in direct binding studies with [^{3}H]-BTX-B and a large sample of local anesthetics (11). In general, local anesthetic potency was linearly correlated with the ability to displace specifically-bound [^{3}H]-BTX-B using guinea pig synaptoneurosomes in a competitive binding paradigm. The allosteric nature of local anesthetic displacement of [^{3}H]-BTX-B binding in rat brain synaptosomes has been recently corroborated by Postma and Catterall (15). The observed inhibition is due to an increase in the off rate for BTX-B binding, thus decreasing the binding affinity, with no effect on the maximum number of binding sites.

Synthetic pyrethroids, structurally based upon esters of the naturally-occurring chrysanthemic acid (2,2-dimethyl-3-(2-methylpropenyl)cyclopropane carboxylic acid) have found widespread use as potent insecticides (16,17)). These compounds are also known to have profound neurotoxic effects in mammals (18,19). Electrophysiological studies, carried out primarily in non-mammalian systems, have provided strong evidence for an effect of pyrethroids on the voltage-sensitive sodium channel (20-23). Voltage clamp experiments reveal the presence of a depolarizing afterpotential in pyrethroid-treated nerve fibers resulting from a decrease in the rate of sodium channel inactivation. Additional data supporting a sodium channel site as a primary target for pyrethroids in mammalian neuronal tissue have been reported. Jacques et al. (24) observed a synergistic enhancement by some pyrethroids of ^{22}Na$^+$ uptake mediated by the sodium channel activators veratridine, batrachotoxin, scorpion toxin and sea anemone toxin in mouse neuroblastoma cells. Similar results were reported by Ghiasuddin and Soderlund (25) using a rat brain synaptosomal preparation . These workers also found that the pyrethroid-induced enhancement was stereospecific in that the non-toxic cyclopropane C1-S isomers were without effect. In our laboratory we have been able to demonstrate directly the positively cooperative interaction between several site 4 pyrethroids, including deltamethrin and the four active stereoisomers of cypermethrin, and the binding of [^{3}H]BTX-B to sodium

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channels both in synaptoneurosomes from mouse cerebral cortex and in rat brain synaptosomes (26 and Brown et al., manuscript in preparation). Deltamethrin increases the affinity of [^{3}H]BTX-B binding 3-fold in the presence of saturating concentrations of scorpion toxin. The half maximal concentration for enhancement of toxin binding is 1 μM.

For many years, sodium channel sites 1 and 2 have been considered to be distinct and non-interacting, based primarily on the inability of BTX to affect the binding of TTX or STX (27,28). However, we have shown that, under certain conditions, an allosteric relationship can be observed between the BTX and TTX/STX site manifest as a TTX/STX-induced decrease in BTX-B binding affinity (29). The principal findings are that a) occupancy of the TTX site decreases the affinity of BTX-B binding with no change in the maximum binding capacity, b) the effect is strongly temperature dependent, being negligible at 37°C and most pronounced at 18°C, the lowest temperature investigated, and c) occupancy of the BTX site has no effect on the binding of TTX or STX. At 25°C, in the presence of 1 μM TTX, the dissociation constant for [^{3}H[BTX-B binding to sodium channels in mouse cerebral cortex synaptoneurosomes or rat brain synaptosomes is reduced by a factor of 3-4 relative to that in the absence of TTX.

In contrast to these interactions, a lack of interaction between channel site 6, binding the ß-scorpion toxins, and sites 1,2 and 3 has been reported (30). In our laboratory we have failed to find any effect of ß-toxin IV from the scorpion *Centruroides sculpturatus* on the specific binding of ^{3}[H]BTX-B in rat brain synaptoneurosomes.

Summarizing the discussion above, it is apparent that in addition to its usefulness to probe its own binding site 2, ^{3}[H]BTX-B may also be used to monitor interactions of a wide variety of other sodium channel ligands acting at separate and distinct binding domains by virtue of the allosteric interactions between these sites. As will be described, it should be possible, under carefully selected conditions, to use this phenomenon to advantage in the development of a general screening procedure for the identification of potentially therapeutic compounds.

Relatively little information is available in the literature concerning compounds that may offer potential therapeutic advantage in cases of sodium channel neurotoxin intoxication. Barhanin et al. (31) reported that a chemically modified sea anemone toxin could still bind to its receptor site on the sodium channel protein but had lost all ability to modify channel function. The ability of this derivative to act as an antagonist to unmodified sea anemone toxin was noted, but not elaborated upon. Creveling et al. (11) found that the local anesthetic lidocaine ethiodide was 10 times more potent at displacing specifically bound [^{3}H]BTX-B than in producing block of sodium channel flux. Interestingly, Clarkson and Hondeghem (32) report that lidocaine was able to attenuate the effects of bupivacaine, a more potent local anesthetic, in voltage-clamped guinea pig papillary muscle. Noting that bupivacaine toxicity can be a serious and even lethal condition, these authors suggested the therapeutic potential for reducing antiarrhythmic drug toxicity by displacement with other drugs. We have found that batrachotoxinin-A, a minor component of the alkaloid mixture containing BTX, exhibits only 50-fold less binding affinity for sodium channel site 2 even though this compound is 1/1000 as toxic as BTX (7).

Regarding pyrethroid toxins, we have shown that several non-toxic stereoisomers of cypermethrin can attenuate the effects of active pyrethroids on the binding of ^{3}[H]BTX-B (26). Of further interest is the observation that several relatively simple pyrimidine and benzimidazole derivatives, synthesized as limited analogs of the channel blockers TTX and STX, were relatively weak blockers of sodium flux(33), yet we have found one of these, HM-197, to be a rather potent inhibitor of ^{3}[H]BTX-B binding (Brown, unpublished results). These observations all suggest that the potential exists to develop effective therapeutic measures to counter sodium channel intoxication, but to our knowledge this approach has not been pursued in a systematic way.

RATIONALE

The initial goal of these studies has been to develop a screening mechanism that can be applied in a systematic way to the identification of compounds with potential therapeutic value in the treatment of neurotoxin poisoning. As noted above, of the possible therapeutic approaches, one with apparently high potential for success involves the identification of compounds that by an interaction with the sodium channel or the toxin bound to the sodium channel either counteract or eliminate the effects of the toxic agent. Thus, one prerequisite for advancing this approach is a means to identify compounds that meet the first criterion, namely the ability to interact with the sodium channel protein or its ligands. In order to be useful and practical, we have considered that the screening assay should a) be able to accommodate large numbers of samples, b) be reproducible, simple to use, and amenable to performance in any moderately equipped biochemistry laboratory, and c) be as general as possible with respect to detection of interactions at multiple sodium channel sites or toxins in one step. The first two requirements can be met in general by a radioligand binding assay, and our results on the pharmacology of BTX-B suggest that this ligand can provide the needed generality since its binding is influenced by interactions at five of six well-studied sodium channel binding domains. The immediate objective of these studies is therefore to define conditions of a [^{3}H]BTX-B radioligand binding assay wherein the amount of specific [^{3}H]BTX-B bound serves as a sensitive indicator of test compound interactions at any of the several sodium channel sites allosterically coupled to the BTX site as well as at the BTX site itself. In order to accomplish this we have investigated the inclusion of several non-radioactive neurotoxins in the assay medium to confer a delicately balanced level of [^{3}H]BTX-B binding in the absence of test compounds which can respond sensitively to the addition of those compounds with either an increase or decrease in binding, depending on the pharmacological activity and the particular site of action.

Another consideration affecting the ease of use of the screening assay as well as its sensitivity and stability concerns the tissue preparation used as a source of sodium channels. A microvesicular preparation from rat cerebral cortex, termed synaptoneurosomes, was chosen since it contains a reasonably good density of sodium channels (2 pmoles/mg protein), maintains a nominal membrane potential that is necessary for high affinity BTX-B binding in the presence of scorpion toxin, and can be prepared more easily and quickly than the conventional synaptosome preparation. To further simplify application of the screening assay, we considered that it would be helpful and

efficient to be able to prepare synaptoneurosomes in bulk and store aliquots for use over extended periods of time. Conditions of storage that maintain the stability of the preparation have therefore been investigated.

Since the assay is designed to detect interactions at the sodium channel sites in general, potential "neurotoxins" as well as potential therapeutic agents might be identified. Once active compounds have been flagged, their therapeutic value must be tested more directly with other techniques, as this information will not be immediately recovered from the screening assay. For a front line, general screening procedure, however, we consider this aspect an advantage, since discovery of new neurotoxins could add to the database of structure-activity relationships at the sodium channel and complement the search for effective treatment modalities. For further testing of compounds which are identified in the screening assay, we have applied an electrophysiological method which allows measurement of effects on nerve and muscle compound action potentials (as well as on neuromuscular transmission and muscle contraction) in a single preparation. In addition, we have incorporated a [^{3}H]STX binding assay using screening assay conditions to permit more directed measurements of interactions at that particular site. These steps have placed us in a position both to validate the effectiveness of the screening assay per se and to follow up on promising candidates for therapeutic intervention as they are flagged by the screening assay.

EXPERIMENTAL METHODS

A. Buffers. HEPES buffer was composed of 130 mM choline chloride, 50 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfornic acid), 5.5 mM glucose, 0.8 mM $MgSO_4$, and 5.4 mM KCl, adjusted to pH 7.4 with Trizma base. WASH buffer was composed of 163 mM choline chloride, 5mM HEPES, 1.8 mM $CaCl_2$, and 0.8 mM $MgSO_4$ adjusted to pH 7.4 with Trizma base. TES buffer consisted of 20 mM TES (N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid), 112 mM NaCl, 2.5 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 15 mM $NaHCO_3$, 0.4 mM KH_2PO_4, and 10 mM glucose adjusted to pH 7.4 with NaOH. Modified TYRODE'S solution contained 135 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM Na_2HPO_4, 15 mM $NaHCO_3$, and 11 mM glucose, pH 7.4. Isotonic SUCROSE solution consisted of 0.32 M sucrose, 10 mM NaH_2PO_4, pH 7.4.

B. Synaptoneurosomes. Synaptoneurosomes were prepared by a modification of the procedure described by Creveling et al. (11). Briefly, freshly dissected cerebral cortical tissue from male Sprague-Dawley rats was homogenized in 2 volumes (wt/vol) of ice cold HEPES buffer using 10 strokes of a loose-fitting glass-glass homogenizer. The homogenate was diluted with 2 additional volumes of cold buffer and centrifuged at 1000xg for 15 min at 5°C. The supernatant was discarded and the pellet was resuspended in HEPES buffer (or other buffers as indicated) by repetitive pipetting with a 9 inch Pasteur pipette or by gentle homogenization in a loose-fitting glass-glass homogenizer. This preparations was used directly as "unfiltered synaptoneurosomes". For preparation of "filtered synaptoneurosomes", the pellet from the 1000xg centrifugation was resuspended

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in 20 volumes of HEPES buffer (original wt/vol) by homogenization using 3 strokes of a loose-fitting glass-glass homogenizer and filtered through a Millipore AP prefilter overlaid with 3 layers of HC-3 160 mesh nylon (Tetko, New York). The filtrate was collected in ice cold tubes and subsequently filtered through a Millipore LCWP 047 filter having a 10 µm cut-off. This filtrate was then centrifuged at 1000xg for 30 min at 5°C, and the pellet resuspended in buffers of choice for use. Protein concentration for both filtered and unfiltered preparations was generally adjusted to approximately 6 mg/ml. Protein determinations were performed using the procedure of Peterson (34) with bovine serum albumin as a standard. Alternatively, protein concentratration was estimated by dilution of 75 µl of the tissue suspension in 1.5 ml distilled water and recording the absorbance at 280 nm (uncorrected for light scattering). The absorbance reading was converted to protein concentration with reference to a standard curve calibrated against protein concentrations determined with the Peterson procedure.

C. Measurement of tritiated toxin binding. Measurement of specific [^{3}H]BTX-B binding was performed as reported previously (29). Briefly, standard binding reactions were initiated by addition of 150 µl of synaptoneurosome suspension in HEPES buffer containing approximately 1 mg protein to a solution in HEPES buffer of [^{3}H]BTX-B and various concentrations of unlabeled effectors as indicated. The concentration of labeled toxin was generally 8-9 nM, and the total assay volume was 320 µl. The effectors were added from concentrated stock solutions (scorpion toxin, 2 mg/ml water; TTX, 100 µM in water; veratridine, 10 mM in MeOH;, pyrethroids, 5 mM in MeOH; all others 10 mM in water), Incubations were carried out for 60 min at room temperature and were then terminated by addition of 3 ml cold WASH buffer. The tiss was immediately collected on Whatman GF/C glass fiber filters using a Brandel 30-place filtration manifold, and washed 3 more times with 3 ml cold WASH buffer. Radioactivity associated with the tissue was determined by liquid scintillation spectroscopy of the filters suspended in 10 ml scintillation cocktail (3a70B, RPI). Nonspecific binding was determined from parallel assays containing 250 µM veratridine and has been subtracted from the data. Binding of [^{3}H]STX was measured in a manner analogous to that described for [^{3}H]BTX-B. In these assays, the concentration of [^{3}H]STX was adjusted to approximately 1 nM, and unlabeled TTX or STX was included in parallel assays for the determination of non-specific binding.

Data points were determined in triplicate and are presented as the mean ± S.D. of these determinations. Unless otherwise indicated, figures show the results of 1 experiment that is representative of 2 or more separate determinations.

D. Fluorescent measurement of relative membrane potential. Freshly prepared synaptoneurosomes and synaptoneurosomes arising from various treatments (frozen storage) were diluted into TES buffer to a concentration of approximately 3 mg protein/ml. One hundred microliters of these suspensions were added to cuvettes containing 1.8 ml of a solution of 3-3'-diethyloxadicarbocyanine (80 µM) in TES buffer and allowed to equilibrate while monitoring fluorescence at 600 nm (580 nm excitation). After a stable baseline had been achieved (approximately 3 min), 50 µl of a 4 M KCl solution was added to give a concentration of 102 mM, leading to depolarization of the synaptoneurosomes. The resulting increase in fluorescence intensity, ΔF, was recorded for fresh

synaptoneurosomes and test samples. The relative membrane potential of the test samples was then expressed as a percentage of that of the fresh synaptoneurosomes by the expression $(\Delta F_t/\Delta F_f) \times 100$. Previous work has shown that the fluorescence response of the dye is a linear function of membrane potential in this system (35).

E. _Electrophysiological measurements._ The diaphragm and phrenic nerve were dissected from 150 g rats under pentobarbital anesthesia and a 5-10 mm diameter strip of muscle with nerve attached was suspended in an apparatus permitting measurement of the nerve compound action potential. The apparatus is a modification of that described earlier by Pagala (36). The muscle was fixed at the bottom of a glass chamber and connected to a force transducer at the top to measure muscle tension. Two pairs of platinum electrodes contacted the phrenic nerve for stimulation and recording of the nerve compound action potential. The muscle could be stimulated directly _via_ two plate electrodes and the muscle compound action potential recorder by another platinum electrode situated just below the top tendon. (See Figure 7). The entire arrangement was immersed in TYRODE'S solution and bubbled continually with 95% O_2 and 5% CO_2. The apparatus was surrounded by a thermostatted circulating water jacket and the temperature controlled at 30°C. In order to collect the data presented here, the solution level in the apparatus was lowered below the level of nerve insertion in the muscle, and the distal nerve electrodes used to deliver supramaximal stimulating square wave pulses of 0.1 msec duration at a frequency of 100 Hz for 1 sec unless otherwise indicated. The resulting train of nerve compound action potential recorded at the proximal pair of electrodes was displayed on a storage oscilloscope and photographed with Polaroid film to produce a permanent record. Following these brief recording periods, the fluid level in the bath was restored to full height. Control experiments have demonstrated that, under these conditions, reproducible responses of the preparation may be obtained over a period of 5-6 hours.

RESULTS

Conditions for preparation and storage of synaptoneurosomes.

Several variables with potential to impact the integrity of the synaptoneurosomes upon long-term frozen storage and preparation for assay were investigated. These included the use of filtered or unfiltered synaptoneurosomes, several different buffers for frozen storage, including HEPES, HEPES made 12% in dimethylsulfoxide, and SUCROSE buffers, and various procedures for thawing of the synaptoneurosomes prior to assay, such as thawing at 5°C, thawing at room temperature, and the inclusion of a 15 min preincubation at 37°C after thawing but prior to assay. Almost all permutations of these variable were assessed. In many experiments, a cocktail of protease inhibitors was included in the buffers from start to finish, including pepstatin A (0.5 µM), iodoacetamide (1 mM), and phenylmethanesulfonyl fluoride (0.1 mM). In no case did these agents yield any improvement in synaptoneurosome stability, and they were not further investigated. Stability of the synaptoneurosomes following these various treatments was assessed by two separate measurements. Since high affinity binding of BTX-B depends upon allosteric interactions with bound scorpion toxin, and scorpion toxin binding is membrane

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potential-dependent (37), the relative membrane potential was determined using the fluorescence assay in addition to measuring the specific binding of BTX-B in the presence of a saturating concentration of *L. quinquestriatus* scorpion venom. These measurements were compared to those obtained in parallel assays using freshly prepared synaptoneurosomes to define conditions which would allow bulk preparation of tissue and frozen storage of aliquots in order to increase the practicality and ease of use of the screening assay. Figure 1 shows the results obtained with several different parameters, including the use of filtered synaptoneurosomes frozen in SUCROSE buffer and thawed at 5°C prior to use, a procedure which gave excellent retention of both membrane potential and BTX-B binding. These conditions were therefore selected for more extensive testing with respect to the maximum permissible time of storage. Note the close correspondence in Figure 1 between the relative membrane potential and BTX-B binding, suggesting that the ability to establish a nominal membrane potential following storage and thawing is critical.

In order to test the stability of the tissue preparation in SUCROSE buffer to longer-term storage, filtered synaptoneurosomes were prepared according the the protocol outlined in Figure 2. One aliquot was assayed immediately to define the original level of specific BTX-B binding, and the remaining aliquots were stored frozen at -70°C. At monthly intervals, an aliquot of tissue was removed from storage, thawed at 5°C, collected by centrifugation at 1000xg for 15 min at 5°C, and finally resuspended in HEPES buffer at a concentration of 6 mg protein/ml for measurement of specific BTX-B binding and comparison to the original level. In these experiments, BTX-B binding was measured under standard screening assay conditions as described below. that is in the presence of other unlabeled neurotoxins, to reflect conditions that would actually be used in an application of the screening assay. The results are shown in Figure 3. The amount of specific binding remaining after 5 and 6 months of storage was approximately 50% of the original value. In absolute terms, this means that there was still about 600 cpm of specific binding in the face of 1500-1600 cpm of total binding (see below). Even though this amount of specific binding is sufficient for operation of the screening assay, in the practical sense we recommend that the tissue be stored no longer than 4 months, at which time 70-80% of the original specific binding remains.

The integrity of the TTX/STX site in frozen synaptoneurosomes was also tested. Synaptoneurosomes which had been stored for 3 months in SUCROSE buffer were prepared as above for a binding assay in which the ability of unlabeled TTX to inhibit the binding of a tracer amount of [^{3}H]STX as a function of concentration was determined in the absence of other sodium channel neurotoxins. As shown in Figure 4, TTX inhibits binding with an apparent dissociation constant of 17 nM, in good agreement with previous results (29). This experiment demonstrates that this assay can be used directly as an adjunct to the standard screening assay in order to test for interactions at site 1 specifically without altering requirements for preparation or storage of the tissue.

Standard screening assay conditions.

Complete dose-response curves for the effects of TTX, deltamethrin, and α-scorpion toxin alone on specific [^{3}H]BTX-B binding were examined using filtered

synaptoneurosomes. The curves were then repeated for each unlabeled effector in the presence of half-maximal concentrations of the other effectors as determined from the first set of experiments. Based on these results, the following concentrations of the unlabeled effectors were selected for inclusion in the standard screening assay: TTX, 25 nM; deltamethrin, 1 µM; scorpion toxin, 2.5 µg/ml of the lyophylized venom of *L. quinquestriatus*. These concentrations were selected to produce approximately half-maximal effects mediated through these different sodium channel sites in the assay. Thus, the binding of [^{3}H]BTX-B should be highly sensitive to allosteric modulation induced by either agonist or antagonist properties of test compounds acting at these sites. In the absence of any test compounds and in the presence of unlabeled toxins at the indicated concentrations, equilibrium binding of 9 nM [^{3}H]BTX-B to synaptoneurosomes at a concentration of 3 mg protein/ml yields approximately 4000 dpm of total binding, of which 50% is specific.

We considered the possibility that, in addition to synaptoneurosomes, aliquots of the toxins at concentrations appropriate for the assay might be prepared in advance and stored frozen. As shown in Figure 5, however, testing of the toxins after 3 weeks of storage at -70°C in HEPES buffer revealed significant instability of both scorpion toxin and BTX-B to these conditions. It is therefore not possible to simplify the set-up of the screening assay using this approach.

Initial tests of the screening assay.

Several compounds were selected for initial testing of the screening assay response based upon their known ability to interact at the sodium channel and their potential therapeutic interest as discussed under Background. To date, five compounds have been included in the standard screening assay procedure at several concentrations in order to see if their predicted dose-dependent inhibitory effects on BTX-B binding could be detected by the assay as presently constituted. The results of these tests are presented in Table I. It is clear that each of these agents would be identified as a sodium channel effector under normal operation of the screening assay as long as the appropriate concentration range was included in the study.

One of the most interesting agents in this series is HM-197, 5-benzoyloxy-2-iminohexahydropyrimidine. As mentioned, HM-197 was first prepared as a simple TTX/STX analog and was found to block sodium channels at low millimolar concentrations (33). In order to see if HM-197 was acting at site 1 of the sodium channel, or at some other site, complete dose response curves for inhibition of both STX and BTX-B binding in the absence of other effectors was obtained. These results are presented graphically in Figure 6, showing that HM-197 has little effect on STX binding even at concentrations as high as 400 µM, while the half-maximal concentration for inhibition of BTX-B binding is 35 µM. Thus the effect of HM-197 is not mediated through an interaction at the STX/TTX binding site.

Electrophysiological testing of HM-197.

In view of the results described above, HM-197 was selected for further evaluation of therapeutic potential using the apparatus depicted in Figure 7. The experiments were designed to test the effect of HM-197 alone on the nerve compound action potential and the ability of HM-197 to protect or rescue the nerve from the depolarizing effect of veratridine as suggested by the binding experiments. Veratridine was substituted for batrachotoxin in these experiments because it is relatively inexpensive and available in quantity, in contrast to batrachotoxin. A rat phrenic nerve-diaphragm preparation in Tyrodes solution was mounted in the chamber as indicated in Figure 7 and incubated at 30 °C for varying periods of time with 50, 200, or 600 µM HM-197. The response of the nerve to a single stimulation or stimulation at 100 Hz (0.5 msec stimulus duration) for one second was compared to control responses (without HM-197). At 50 µM, HM-197 had no effect on the action potential up to 60 min. With 200 µM, the action potential was unchanged after 30 min incubation, but slightly reduced in height after 60 min (25%). This effect was reversed completely within 60 min of washing. Washing was accomplished by withdrawing the test solution through the port in the bottom of the chamber and introducing several changes of fresh TYRODE'S solution. Incubation with 600 µM HM-197 for 30 min and 60 min led to reductions in the action potential height of approximately 50 and 60%, respectively. These effects were reversed with a one hour wash period. We also noted a slight decrementing response during the first quarter of the stimulus train apparently induced by HM-197.

As expected, veratridine at a concentration of 7 µM almost completely abolished the action potential within 60 min of incubation. At 30 min of incubation, a marked decrementing response could be seen throughout the stimulus train. The action potential did not completely recover even after two hours of washout. If HM-197 at a concentration of 50 µM was added to a preparation which had been depolarized by prior 30 min incubation with veratridine, the effect of veratridine was not blocked or reversed as shown by the lack of an action potential response at 30 min post HM-197 (60 min post veratridine). In this case however, a two hour wash period did result in significant recovery of the action potential.

Although HM-197 failed to reverse veratridine-induced depolarization when added after administration of veratridine, co-administration of these agents did reveal significant protection by HM-197. This result is shown in Figure 8. Figure 8 A presents the control response of the nerve to the stimulus train. The response after 60 min incubation with 7 µM veratridine alone is shown in Figure 8 B, and the response after 60 min co-incubation with 7 µM veratridine and 50 µM HM-197 is demonstrated in Figure 8 C. These results corroborate the findings from the binding studies, and indicate that at concentrations where it alone has no detrimental effects, HM-197 may provide protection against the depolarizing effects of toxins acting at site 2 of the sodium channel.

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DISCUSSION

The search for compounds with potential value as therapeutic agents active in cases of intoxication induced by neurotoxins directed against the voltage-sensitive sodium channel would be greatly aided by a screening mechanism of broad scope and sensitivity. Based on the known pharmacology of batrachotoxin, we have developed a means by which compounds acting at a minimum of five different binding domains on the sodium channel can be identified in a single assay. Using a radioligand binding paradigm, a tracer amount of [^{3}H]BTX-B is equilibrated with a vesicular preparation from rat brain in the presence of TTX, deltamethrin, and α-scorpion toxin from *L. quinquestriatus*, each at a concentration approximately at the mid-point of the respective dose-response curve for allosteric inhibition (TTX) or enhancement (deltamethrin and scorpion toxin) of BTX-B binding. By selecting these particular concentrations, the level of BTX-B binding in control assays is in a delicate balance and highly sensitive to the presence of agents added in test assays which might act at any of these sites. This assay can thus detect agonists as well as antagonists, and additional information would be required to determine the relative therapeutic value of compounds identified as potential candidates in the screening assay. The great advantage of this approach, however, is that large numbers of test compounds may be rapidly screened as a first step. Since the time required from set-up of the assay to initiation of counting is only about 2 hr and 30 samples can be filtered simultaneously with the aid of a manifold, the efficiency of the assay is clearly great. Compounds that, by themselves, have little or no effect on sodium channel function yet bind with relatively high affinity to a particular sodium channel site represent one class of potential therapeutic agents since they might be efficacious in antagonizing the actions of a neurotoxin. While it could be difficult to identify such compounds directly by electrophysiological techniques, they would be found using the [^{3}H]BTX-B screening assay.

Synaptoneurosomes from rat cerebral cortex were chosen as the tissue preparation for the screening assay because the density of sodium channels approximates that of synaptosomes (38), they maintain a nominal membrane potential necessary for scorpion toxin binding and hence high affinity BTX-B binding (11), and they are prepared more easily and with less time than the traditional synaptosome preparation. We have also demonstrated that the "filtered" synaptoneurosomes may be prepared in bulk and stored frozen at -70°C in buffered isotonic sucrose solution with good stability over a four month period, thus further increasing the simplicity of routine application of the assay. These features contribute to the practicality of the screening assay. Unfortunately, the stability of the neurotoxin cocktail utilized in the assay to frozen storage was found to be inadequate for routine use.

As a preliminary test of the assay validity, we have to date investigated the effects of five test compounds under conditions that would normally be used in running the assay. Based on existing evidence, it was anticipated that several of these compounds would give a positive response. As discussed, the simple TTX analog HM-197 was selected since we had previously found it to inhibit BTX-B binding at relatively low micromolar concentrations (Brown, unpublished observations), whereas blockade of sodium channels, presumbly at site 1, was reported to occur at much higher concentrations (33).

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Benzimidazole and 2-ß-aminoethylbenzimidazole were also included in this group as potentially interesting TTX analogs. The cypermethrin stereoisomers CGA-98496 and CGA-98479 were chosen based on their known ability to antagonize the positively cooperative allosteric effect of the potent insecticide deltamethrin on BTX-B binding to sodium channels even though they themselves are inactive insecticides (26). As predicted, the results in Table I show that HM-197 and the two pyrethroids induced significant reductions in BTX-B binding at low micromolar concentrations. The benzimidazoles were much less potent.

Further testing of HM-197 led to the surprising conclusion that its effects were not mediated through an interaction with site 1 of the sodium channel. Since HM-197 was synthesized as a TTX analog and Brown (29) has shown that TTX and STX may allosterically inhibit the binding of BTX-B, we suspected that HM-197 in the assay was indeed acting at site 1. This was not borne out by direct investigation of the ability of HM-197 to displace specifically-bound [^{3}H]STX. Electrophysiological studies of HM-197 showed that this compound was able to antagonize veratridine-induced depolarization of the rat phrenic nerve at concentrations where it alone had no effect on the action potential. We therefore suggest that HM-197 should be classified as a potential therapeutic agent and subjected to further study. Additional pyrimidine derivatives are currently under investigation in our laboratory, including THP and THP-OH supplied by Dr. B.J. Gabrielsen, U.S. Army Medical Research Institute of Infectious Dieseases. Studies are also continuing in our laboratory to determine the site of action of HM-197.

In summary, we have described a sensitive, simple and rapid screening assay of broad scope which can be used to advantage in the identification of potentially therapeutic compounds active at the mammalian voltage-sensitive sodium channel. Initial testing of the screening procedure to date has provided evidence of suitability for the intended purpose and has as well resulted in the identification of a new line of research to evaluate the potential of various pyrimidine derivatives as therapeutic agents for site 2 intoxication.

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Agent	Concentration (μM)	Mean % Specific BTX-B Binding ± S.D (n=3)
HM-197	10	73 ± 22
	50	47 ± 5
Benzimidazole	100	95 ± 4
	400	66 ± 5
2-ß-aminoethyl-benzimidazole	100	96 ± 11
	400	38 ± 1
CGA- 98496	1	56 ± 3
	10	34 ± 1
	50	22 ± 6
CGA-98479	1	61 ± 9
	10	15 ± 3
	50	3 ± 2

Table I. Effect of test agents in the screening assay

The compounds above were incubated at the indicated concentrations with synaptoneurosomes under the standard screening assay conditions described in the text. Specific binding of [³H]BTX-B was determined and expressed as a percentage of specific binding in parallel incubations without these compounds.

KEY

☐ Fresh tissue control

▨ Standard binding medium, room temp. thaw, unfiltered synaptoneurosomes

◩ Standard binding medium plus DMSO, room temp thaw, unfiltered synaptoneurosomes

▤ 0.32M sucrose, room temp thaw, unfiltered synaptoneurosomes

▨ 0.32M sucrose, 5° thaw, filtered synaptoneurosomes

▨ 0.32M sucrose, 5° thaw, 37° pre-incubation, filtered synaptoneurosomes

■ Paired fluorescence signal

Figure 1. Conditions affecting stability of synaptoneurosomes to frozen storage at -70°C.

Filtered or unfiltered synaptosneurosomes prepared in the buffers indicated above were stored for three weeks at -70°C, thawed as indicated and used for determinations of membrane potential and amount of specific BTX-B binding relative to that in freshly prepared synaptoneurosomes. Standard incubation medium refers to HEPES buffer, and standard incubation medium plus DMSO refers to HEPES buffer made 12% in dimethylsulfoxide. Following thawing, the tissue was washed and collected by centrifugation and resuspended in HEPES buffer for the assays as described under Experimental Procedures. Specific BTX-B binding was measured in the presence of a saturating concentration of scorpion toxin. Binding data are reported as the mean of triplicate determinations, whereas the fluorescence measurements are the result of a single determination for each condition.

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┌─────────────────────────┐
│   Rat Cerebral Cortex   │
└─────────────────────────┘
             │
┌─────────────────────────────────────┐
│ Homogenize in a loose-fitting glass  │
│ glass homogenizer in two volumes     │
│ (w/v) HEPES buffer. Dilute with two  │
│ additional volumes of buffer.        │
└─────────────────────────────────────┘
             │
┌─────────────────────────────────────┐
│ Centrifuge at 1000xg, 5°C, (3500 rpm │
│ in Sorvall with SS-34 rotor) for 15  │
│ min. If the volume per tube is more  │
│ than 10 ml, increase the time        │
│ accordingly.                         │
└─────────────────────────────────────┘
        │                    │
┌──────────────┐      ┌──────────┐
│ Supernatant  │      │  Pellet  │
└──────────────┘      └──────────┘
                           │
              ┌─────────────────────────────────┐
              │ Resuspend pellet in 20 volumes   │
              │ (original weight/v) of HEPES     │
              │ buffer using three strokes of a  │
              │ glass/glass homogenizer.         │
              └─────────────────────────────────┘
                           │
              ┌─────────────────────────────────┐
              │ Filter the tissue through three  │
              │ layers of 160 mesh nylon and a   │
              │ Millipore AP prefilter           │
              │ simultaneously. The prefilter    │
              │ underlies the nylon mesh. More   │
              │ than one prefilter may be        │
              │ required since this filter may   │
              │ clog after an amount of the      │
              │ tissue has been passed.          │
              └─────────────────────────────────┘
                      │               │
              ┌──────────────┐   ┌───────────┐
              │   Filtrate   │   │ Retentate │
              └──────────────┘   └───────────┘
                      │
        ┌───────────────────────────────┐
        │ Filter through a Millipore     │
        │ LCWP 047 filter (10 µm)        │
        └───────────────────────────────┘
            │                 │
    ┌──────────────┐   ┌───────────┐
    │   Filtrate   │   │ Retentate │
    └──────────────┘   └───────────┘
            │
    ┌─────────────────────────────────────┐
    │ Centrifuge at 1000xg for 30 min at   │
    │ 5°C                                  │
    └─────────────────────────────────────┘
        │               │
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│ Supernatant  │   │  Pellet  │
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                        │
    ┌─────────────────────────────────────────┐
    │ Resuspend the pellet in isotonic sucrose │
    │ solution (pH 7.4, 10 mM phosphate         │
    │ buffered). Begin with a 1:6 dilution      │
    │ (original weight of tissue : volume of    │
    │ buffer). 75 µl of this suspension         │
    │ diluted into 1.5 ml water should give an  │
    │ absorbance reading of approximately 1.0   │
    │ at 280 nm. If reading is higher, dilute   │
    │ the 1:6 suspension accordingly with more  │
    │ isotonic sucrose solution.                │
    └─────────────────────────────────────────┘
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    ┌─────────────────────────────────────────┐
    │ Aliquot to cryotubes and freeze at -20°C. │
    │ When frozen, transfer to -70°C freezer    │
    │ for storage up to four months before use  │
    └─────────────────────────────────────────┘
                        │
        ┌─────────────────────────────────┐
        │   Frozen   Synaptoneurosomes    │
        └─────────────────────────────────┘
```
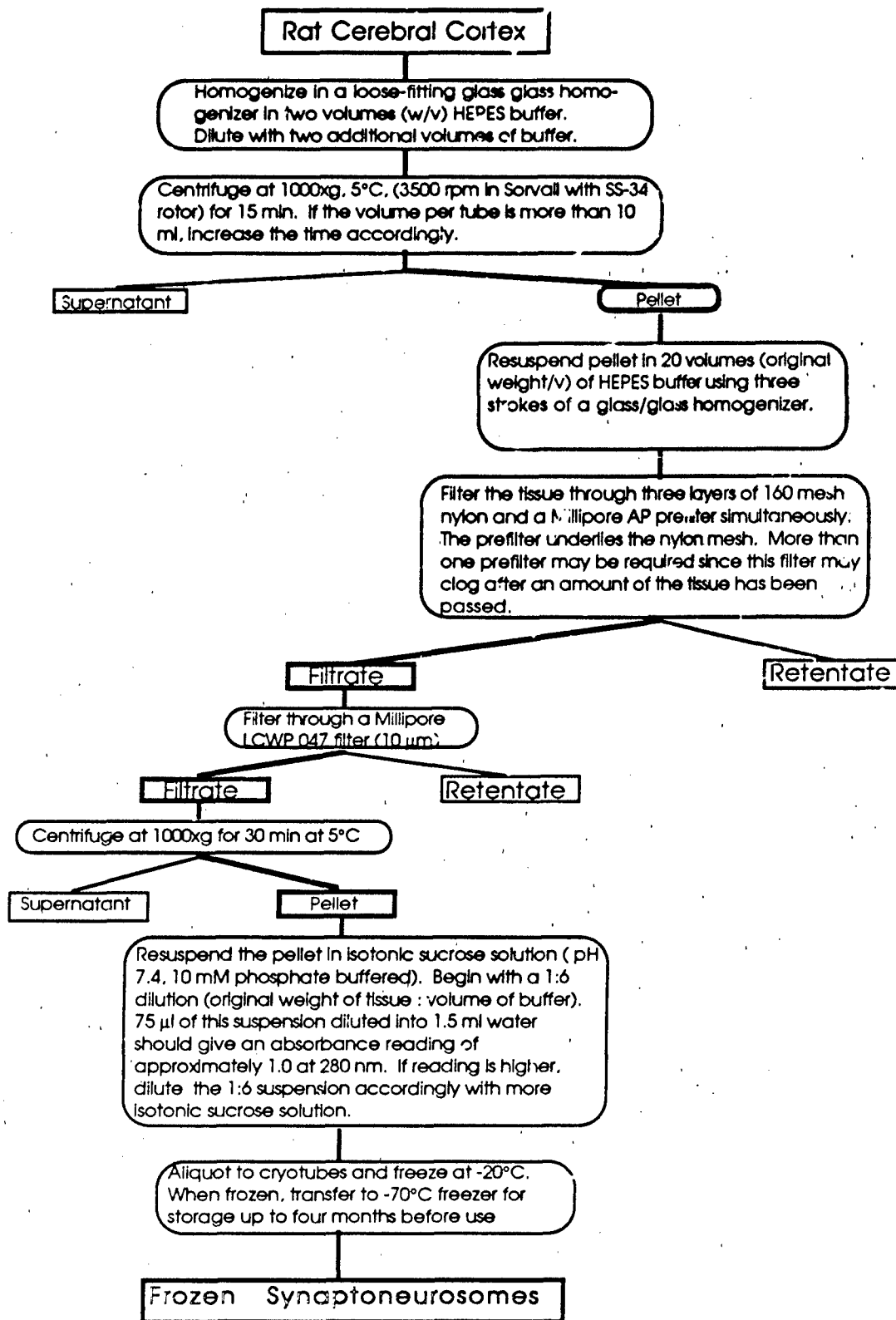
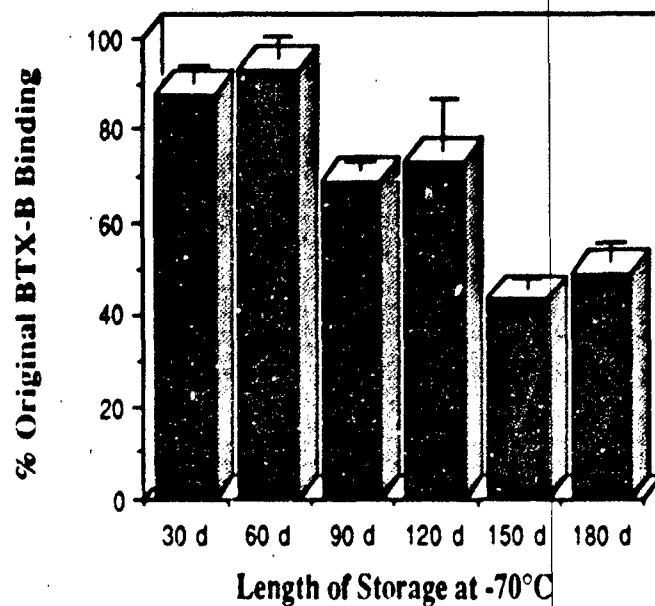Figure 2. Flow-chart for the preparation of filtered synaptoneurosomes

Figure 3. Stability of filtered synaptoneurosomes to extended storage at -70°C.

Synaptoneurosomes were prepared as indicated in Figure 3 and stored in aliquots at -70°C for the periods of time indicated. At monthly intervals, an aliquot was removed and specific binding of BTX-B was measured under standard screening assay conditions. The data are the means ± S.D. of triplicate determinations, expressed as percent specific binding normalized to specific BTX-B binding in the freshly-prepared synaptoneurosomes prior to frozen storage.
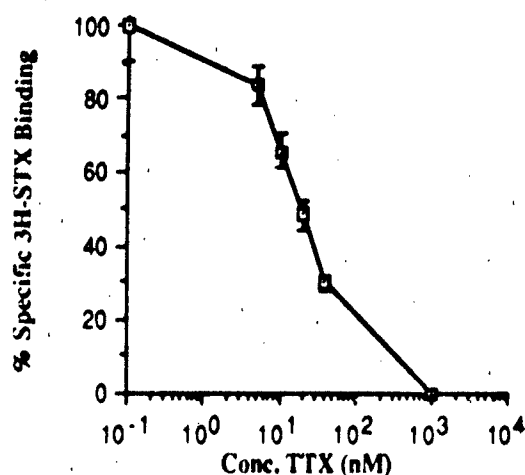
Figure 4. TTX inhibition of [^{3}H]STX binding in frozen synaptoneurosomes

Synaptoneurosomes were thawed at 5°C after three months of storage in isotonic
sucrose buffer at -70°C and used in the assay described in the text to measure the inhibition
of specific [^{3}H]STX binding by TTX. The concentration of [^{3}H]STX was held constant at
1 nM and the concentration of unlabeled TTX varied between 1-1000 nM. Specific binding
at each concentration of TTX is expressed as a percentage of specific binding measured in
the absence of TTX. The approximate equilibrium dissociation constant for TTX binding
determined from this graph is 17 nM. The data points are the means ± S.D. of triplicate
determinations.

KEY

☐ All fresh toxins

☐ Frozen TTX, fresh BTX-B, ScTX, deltamethrin

▨ Frozen deltamethrin, fresh BTX-B, ScTX, TTX

▨ Frozen ScTx, fresh BTX-B, TTX, deltamethrin

▨ Frozen BTX-B, fresh ScTx, TTX, deltamethrin
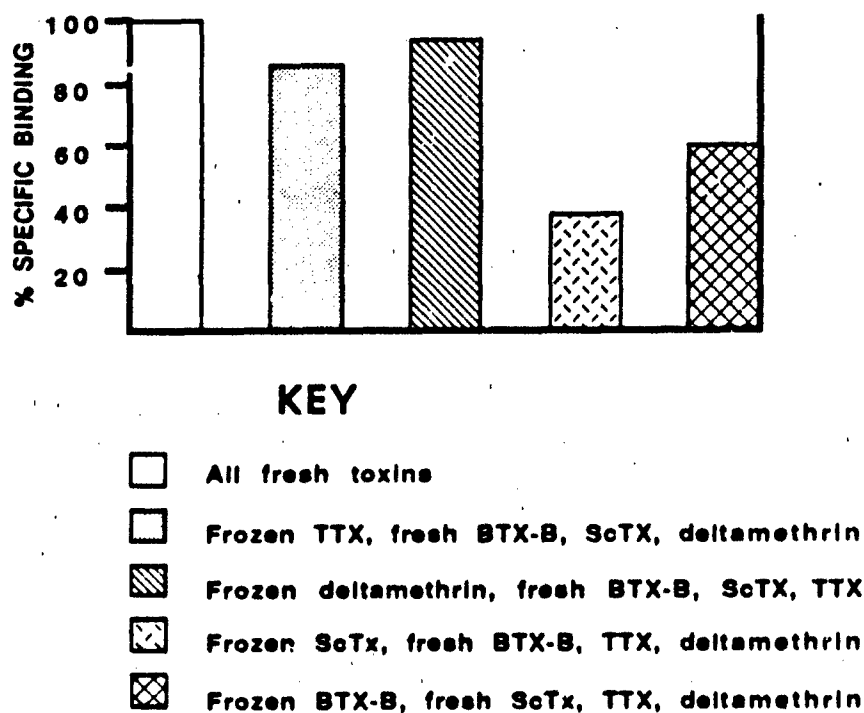
Figure 5. Stability of sodium channel neurotoxins to storage at -70°C

Stock solutions of tetrodotoxin (TTX), scorpion toxin (ScTx), [³H]BTX-B, and deltamethrin in HEPES buffer were frozen and stored at -70°C for 3 weeks. After thawing at 5°C, each toxin was tested by inclusion in a standard screening assay as described in the text along with freshly prepared toxins which had not been frozen. The various permutations tested are indicated in the Key. The specific binding of [³H]BTX-B in each case is expressed as a percentage of binding measured in a parallel assay using all freshly prepared toxins. The data are the means of triplicate determinations.
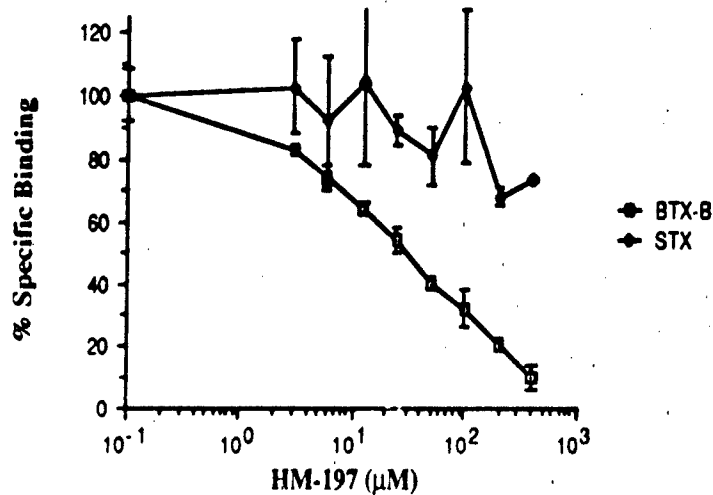
Figure 6. Inhibition of [³H]BTX-B and [³H]STX binding by HM-197

    Binding of [³H]BTX-B in the presence of a saturating concentration of scorpion toxin and of [³H]STX in the absence of other toxins to freshly prepared synaptoneurosomes was measured as a function of concentration of HM-197. The concentration of labeled BTX-B and STX was held constant at 9 nM and 1 nM, respectively. Data are expressed as a percentage of control binding in the absence of HM-197, and are the means ± S.D. of triplicate determinations in a single experiment.
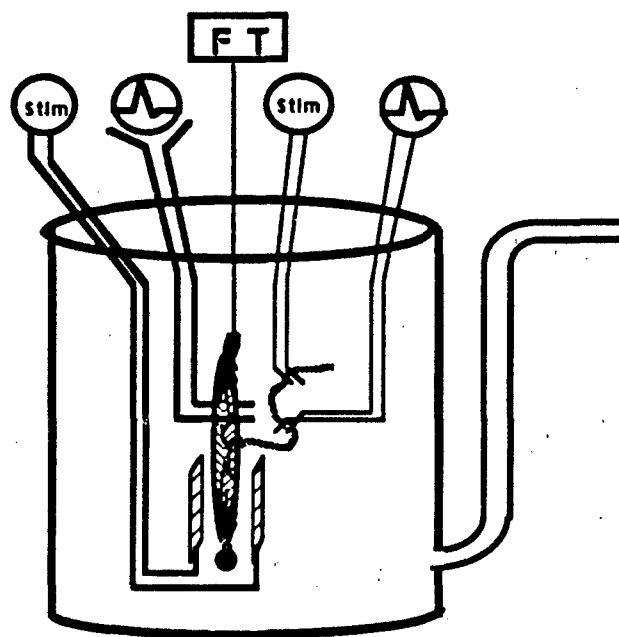
Figure 7.   Nerve-muscle chamber for electrophysiological testing

A freshly-dissected nerve-muscle preparation (typically, rat phrenic nerve-diaphragm) is positioned in the apparatus so that the lower portion of the muscle is suspended between two plate electrodes which are used for direct stimulation of the muscle itself.   The nerve is passed along two sets of electrodes, the distal pair being used for stimulation and the proximal pair for recording of the nerve compound action potential. Muscle compound action potentials elicited either through stimulation of the nerve or direct stimulation of the muscle with the plate electrodes are recorded with the aid of a third pair of electrodes positioned in the upper half of the muscle.   In addition, the muscle is connected at the upper end to a force transducer (FT) for measurement of tension development. The entire assembly is maintained in a beaker containing a buffer (nominally Tyrode's solution) which can be continually bubbled with O_2/CO_2 if required, and to which compounds under investigation can be added. Temperature control is achieved with a thermostatted circulating water bath surrounding the inner beaker. During measurement periods, buffer is withdrawn with a syringe *via* the outlet at the bottom of the beaker until the liquid level is just at the top of the plate electrodes. (The same mechanism may be used to completely withdraw the test solution and introduce fresh buffer to observe the wash-out of effects.) Responses to test stimulations may be recorded on a storage oscilloscope and photographed with a Polaroid camera to produce a permanent record.
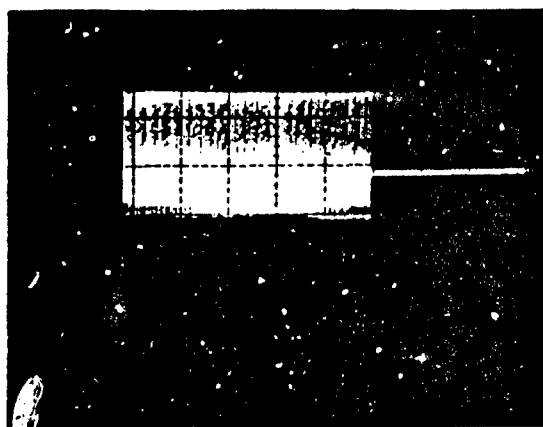
8A
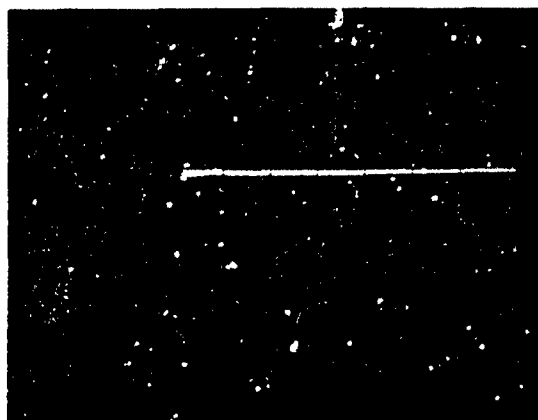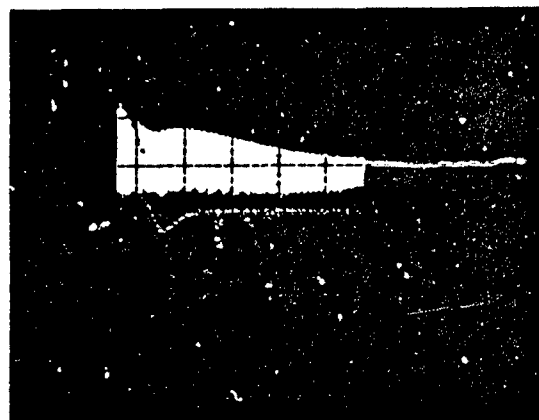
8B   8C

Figure 8. Protection against veratridine-induced depolarization of rat phrenic nerve by HM-197

A rat phrenic nerve-diaphragm preparation was mounted in the chamber depicted in Figure 7 and incubated in Tyrode's solution for 60 min in the absence of any effectors (control, 8A), in the presence of 7 μM veratridine (8B), or in the presence of 7 μM veratridine and 50 μM HM-197 added simultaneously (8C). Following this incubation period, the response of the nerve to a train of supramaximal stimuli (100 Hz, 0.5 msec stimulus duration) was recorded on a storage oscilloscope and photographed with a Polaroid camera. Calibration bars: 2 mV x 0.2 sec.